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—Original Article—

Geminin deletion in pre-meiotic DNA replication stage causes spermatogenesis defect and infertility

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Abstract. Geminin plays a critical role in cell cycle regulation by regulating DNA replication and serves as a transcriptional molecular switch that directs cell fate decisions. Spermatogonia lacking *Geminin* disappear during the initial wave of mitotic proliferation, while geminin is not required for meiotic progression of spermatocytes. It is unclear whether geminin plays a role in pre-meiotic DNA replication in later-stage spermatogonia and their subsequent differentiation. Here, we selectively disrupted *Geminin* in the male germline using the *Stra8-Cre*/*loxP* conditional knockout system. *Geminin*-deficient mice showed atrophic testes and infertility, concomitant with impaired spermatogenesis and reduced sperm motility. The number of undifferentiated spermatogonia and spermatocytes was significantly reduced; the pachytene stage was impaired most severely. Expression of cell proliferation-associated genes was reduced in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes compared to in controls. Increased DNA damage, decreased Cdt1, and increased phosphorylation of Chk1/Chk2 were observed in *Geminin*-deficient germ cells. These results suggest that geminin plays important roles in pre-meiotic DNA replication and subsequent spermatogenesis.
Key words: DNA replication, Geminin, Meiosis, Spermatogenesis

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Geminin (Gmnn) was originally identified in *Xenopus* embryos as a neutralizing molecule that functions during gastrulation and inhibits DNA replication; it is degraded during mitosis [1, 2]. Geminin has dual roles of safeguarding DNA replication and regulating differentiation [3–5]. As an unstable regulator, geminin prevents re-licensing in the G2 phase by preventing loading of minichromosome maintenance (MCM) complexes onto replication origins, a reaction mediated by Cdt1 [6–8]. As cells exit mitosis, geminin is directly ubiquitinated by the anaphase-promoting complex, permitting replication in the succeeding cell cycle [2]. Geminin is also thought to influence the function of the histone acetylase HBO1, which has the essential function of maintaining chromatin in an acetylated state in MCM recruitment [9]. As a transcriptional molecular switch directing cell fate decisions, geminin acts on genes that are targets of specific epigenetic regulators, such as the SWI/SNF chromatin-remodeling complex and members of the repressive Polycomb group [10–12].

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The role of geminin has been examined in various developmental stages. Transient deletion of *Geminin* causes loss of stem cell identity and trophoblast differentiation, which is dependent on intact Brg1 activity [13]. A lack of *Geminin* also leads to preimplantation mortality, concomitant with morphological abnormalities that are responsible for the arrested development of embryos [14]. In the developing neural tube, ablation of *Geminin* during the time window between E8.5 and E10.5, when neural plate patterning and neural tube closure occurs, results in neural tube defects, including decreased differentiation of ventral motor neurons [15]. Our recent study showed that deletion of *Geminin* in mouse oocytes resulted in developmental delay of zygotes [16], with no defects detected in oocyte development, meiotic maturation, ovulation, or fertilization.

Spermatogenesis is a complex developmental process by which male germline stem cells divide and differentiate to produce mature spermatozoa. In mammalian testes, this process occurs within seminiferous tubules and consists of three phases [17, 18]. First, in the proliferative phase, spermatogonia undergo a series of DNA replication cycles as well as mitoses and then differentiate into primary spermatocytes [19]. During the second phase, primary spermatocytes undergo two meiotic divisions to produce haploid spermatids [20]. This phase is subdivided into leptotene, zygotene, pachytene, diplotene, and diakinesis. During the final process of spermatogenesis, which is termed as spermiogenesis, spermatids differentiate into spermatozoa and are released into the lumen of the tubule [21].

The entire process of spermatogenesis is highly coordinated to

protect germ cells against high rates of mutation to maintain genome integrity and involves numerous proteins. Geminin is required for the mitotic self-renewal of spermatogonia but does not regulate spermatocyte meiosis or spermiogenesis, which was evaluated previously by using *Vasa-Cre* and *HspA2-Cre* [22]. However, because of complete germ cell loss during the first wave of spermatogenesis by P4 and because *HspA2-Cre*-mediated recombination begins at the leptotene-zygotene stage [23], the functions of geminin during differentiation from spermatogonia to primary spermatocytes remain unclear.

In the present study, we used the *Stra8-Cre/loxP* system to investigate the functions of geminin in postnatal, premeiotic male germ cells, whose expression begins in the early stage of spermatogonia at P3 and is detected in pre-leptotene spermatocytes [24]. We found that deletion of *Geminin* led to infertility and germ cell defects in both undifferentiated spermatogonia and spermatocytes. Impaired proliferation, increased H2AX phosphorylation, and elevated apoptosis were detected. We also observed decreased Cdt1 and increased Chk1/Chk2 phosphorylation. These results indicate that geminin is required not only for the mitotic proliferation of spermatogonia, but also for pre-meiotic DNA replication and thus spermatocyte meiosis during spermatogenesis.

Materials and Methods

Mice

Geminin flox/flox ($Gmnn^{fl/fl}$) mice were obtained from Jackson Laboratory (016913; Jackson Laboratory, Bar Harbor, ME, USA) [25] and maintained in a 129S4/SvJae; C57BL/6J mixed background. *Stra8-Cre* mice were maintained in a genomic background of C57BL/6J [24]. To improve knockout efficiency, mutant mice were heterozygous for *Stra8-Cre* ($Gmnn^{fl/\Delta}$; *Stra8-Cre*) and control mice were homozygous for *Geminin* floxed allele and *Stra8-Cre*-negative ($Gmnn^{fl/fl}$). Mice were housed with 12-h/12-h light/dark cycles and free access to water and food. All animal operations conformed to the Animal Research Committee of the Institute of Zoology, Chinese Academy of Sciences. DNA extraction from mouse tails was conducted to genotype the $Gmnn^{fl}$ and $Gmnn^{\Delta}$ alleles as well as *Stra8-Cre*. The identification primer pair for $Gmnn^{fl}$ was: 1) 5′-GCTCAGAGGTTTCAGGG-3′, 2) 5′-CATCAGGTGTTCTCTCAAGTGTCTG-3′ and 3) 5′-GCTACTTCCATTTGTCACGTCC-3′. The primer pair for $Gmnn^{\Delta}$ was: 4) 5′-CTAGCCACAGATGTTGAGCTTG-3′ and 5) 5′-CTAGATGGGATGTATTGTATGAGAG-3′. The primer pair for *Stra8-Cre* was: 6) 5′-GTGCAAGCTGAACAACAGGA-3′ and 7) 5′-AGGGACACAGCATTGGAGTC-3′.

Fertility analysis

Fertile $Gmnn^{fl/fl}$ females were mated with 6-week-old $Gmnn^{fl/\Delta}$; *Stra8-Cre* and $Gmnn^{fl/fl}$ siblings. Two females were housed together with one male. The number and size of the litters were recorded for a 6-month period.

Sperm motility analysis

Spermatozoa were extruded from the cauda epididymis and incubated for 15 min in 500 µl Human Tubal Fluid culture medium at 37ºC and 5% CO_2. For sperm motility analysis, a CASA system (Version. 12 CEROS, Hamilton Thorne Research, Beverly, MA, USA) was used.

Immunohistochemistry analysis

Testes were fixed in 4% paraformaldehyde (pH 7.4) at 4ºC overnight, dehydrated in ethanol, and embedded in paraffin. Paraffin-embedded testes were sectioned at a thickness of 5 µm. One or both testes from more than three mice of each genotype were analyzed. After deparaffinization and dehydration, sections were transferred into a sodium citrate solution (0.01 M) in a water bath at 95ºC for 15 min, followed by natural cooling for antigen repair and inactivation of non-specific antigens with 3% H_2O_2 for 10 min. The samples were washed three times with PBS and blocked with 10% donkey serum for 30 min (serum diluted with PBST, PBST: PBS + 0.1% Tween). These sections were incubated with primary antibodies (Sall4, 1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-101147; SCP3, 1:200, Santa Cruz Biotechnology, sc-74569; PCNA, 1:200, Biodragon Immunotechnologies, Beijing, China, B1032) and incubated overnight at 4ºC. The sections were incubated with horseradish peroxidase–conjugated secondary antibodies at 25ºC for 2 h and stained using the DAB chromogenic kit. After staining, the sections were examined with a Nikon microscope and images were captured with a Nikon CCD camera (Nikon, Tokyo, Japan).

Periodic acid Schiff (PAS) staining

Testes were fixed in Bouin's fixative overnight at room temperature, dehydrated in ethanol, and embedded in paraffin. Paraffin-embedded testes were then sectioned at a thickness of 5 µm. After deparaffinization and dehydration, sections were stained with hematoxylin and Periodic acid Schiff for histological analysis. Images were captured with a Leica Aperio Versa 8 (Leica, Wetzlar, Germany). To count the number of spermatocytes, 3 mice were chosen and germ cells were counted in at least 200 seminiferous tubules observed in cross-sections.

Western blot

Each sample containing 200 mg testicular tissue was added to the tissue lysate containing protease and phosphatase inhibitor, followed by 4ºC lysis for 30 min and centrifugation at 12000 rpm for 20 min at 4ºC. The supernatant was mixed with SDS-PAGE buffer and boiled at 100ºC for 5 min. Western blotting was performed as previously described [26] using an antibody dilution of anti-Geminin (Bioworld Technology, MN, USA, BS7535) at 1:100; β-actin (Zhongshan Golden Bridge Biotechnology, Beijing, China, TA-09) at 1:1000; anti-Cdt1 (Millipore, Billerica, MA, USA, 07-1383) at 1:1000; anti–Pi-Chk1S345 (Cell Signaling Technology, Danvers, MA, USA, 2348) at 1:1000; anti-Pi-Chk2T68 (Bioworld, BS4043) at 1:500; and anti–Pi-H2AXS139 (Cell Signaling Technology, 9718) at 1:1000. The membranes were subsequently incubated with horseradish peroxidase–conjugated secondary antibodies (1:2000; ZB2301 and ZB2305; Zhongshan Golden Bridge Biotechnology) for 1 h at 37ºC. Protein bands were detected using the Thermo Supersignal West Pico chemiluminescent substrate (Waltham, MA, USA).

Immunofluorescence analysis

After deparaffinization and dehydration, sections used for staining were transferred into a sodium citrate solution (0.01 M) water bath at

95ºC for 15 min, followed by natural cooling for antigen repair. The membranes were blocked with 10% donkey serum for 30 min (serum diluted with PBST, PBST: PBS + 0.3% Triton X-100), and then reacted with primary antibodies (MVH at 1:500, Abcam, Cambridge, UK, ab13840; Geminin at 1:50, Santa Cruz Biotechnology; Pi-H2AXS139 at 1:200, Cell Signaling Technology) and incubated overnight at 4ºC. Finally, the membranes were incubated for 1 h at 25ºC with a secondary Alexa Fluor 488-conjugated antibody (1:1000, A11008 and A11055, Life Technologies). After three washes, nuclei were stained with DAPI and the sections were mounted. Fluorescence was examined using a laser-scanning confocal microscope (Zeiss LSM 780, Jena, Germany).

Counting Sall4-positive cells

To count the number of Sall4-positive cells, 2 testis sections were scored per animal and at least 3 mice were used. Germ cells were counted in at least 100 seminiferous tubules observed in cross-sections. Tests sections were scored randomly to avoid experimental bias.

TUNEL assay

A TUNEL assay was conducted using the In Situ Cell Death Detection Kit, Fluorescein (Promega BioSciences, Madison, WI, USA) as recommended by the manufacturer. Images were captured using a laser-scanning confocal microscope (Zeiss LSM 780).

Statistical analysis

All experiments were repeated three times. The data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA) by independent sample *t*-test. The data in the graphs are presented as the mean ± standard error (S.E.M.) and were considered significant when $P < 0.05$ (*), 0.01 (**), or 0.001 (***).

Results

Deletion of Geminin gene from male germline

Given that *Geminin*-null mice are early embryonic lethal [14, 27], we crossed $Gmnn^{fl}$ mice in which exons 5–7 were flanked by two loxP sites with transgenic mice expressing *Stra8* promotor-driven Cre recombinase. In *Stra8-Cre* mice, Cre recombinase was only activated in males at day 3 after birth in early-stage spermatogonia and detected in preleptotene spermatocytes [24]. To examine the deletion efficiency of *Geminin*, geminin expression in the testes was examined by western blotting and immunofluorescence. Western blot analysis showed that geminin was notably depleted at the protein level (Fig. 1A). As shown in Fig. 1B, geminin was highly expressed in spermatocytes of control testes, whereas little geminin was detected in those from $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice. These data indicate that geminin was efficiently deleted in $Gmnn^{fl/\Delta}$; *Stra8-Cre* mouse testes.

Selective deletion of Geminin in pre-meiotic germ cells results in germ cell loss and infertility

$Gmnn^{fl/\Delta}$; *Stra8-Cre* mice developed normally with no developmental defects observed until at least 8 months of age. However, adult $Gmnn^{fl/\Delta}$; *Stra8-Cre* males exhibited markedly smaller testes compared to littermate controls (7-week-old, Fig. 2A), and the testis weight of $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice was significantly reduced (Fig. 2B, $P < 0.001$). To investigate the reason for the dramatically decreased testis weight, we performed histological analysis of testes employing immunofluorescence staining. Atrophic tubules and greatly fewer MVH-positive germ cells were observed in $Gmnn^{fl/\Delta}$; *Stra8-Cre* testes (Fig. 2C), indicating that *Geminin* plays a critical role in spermatogenesis and that deletion of this gene results in germ cell loss.

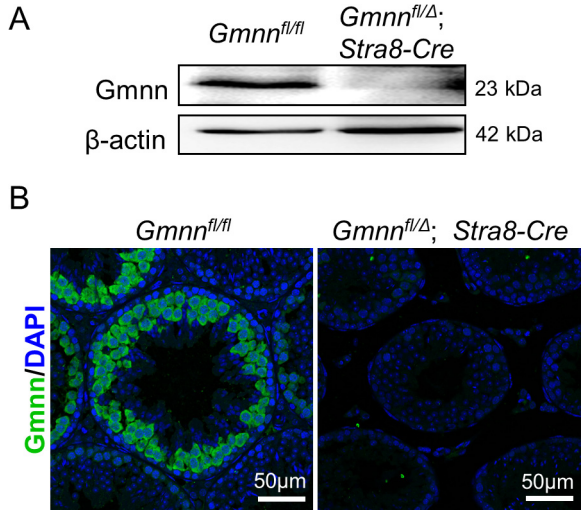

Fig. 1. Deletion efficiency of geminin. A: The protein level of geminin was markedly reduced in $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice. B: Germ cell staining to show deletion of geminin. Geminin (green) in testes; nuclei were stained with DAPI (blue).

To examine $Gmnn^{fl/\Delta}$; *Stra8-Cre* mouse fertility, adult males were mated with wild-type females for 6 months, while $Gmnn^{fl/fl}$ males were used as a control group. Breeding assays showed that $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice were completely infertile (Table 1). Most *Geminin*-deficient males had few spermatozoa in the cauda epididymis. However, a small number of elongating spermatids was observed in both seminiferous tubules (not shown) and the epididymal lumens of $Gmnn^{fl/\Delta}$; *Stra8-Cre* males (Fig. 2D). We therefore used computer-aided sperm analysis (CASA) to investigate the sperm motility characteristics after sperm incubation for 15 min at 37ºC. The percentage of progressive sperm was substantially reduced in $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice compared to in the control group. Sperm motion variables including average path velocity, straight line velocity, curvilinear velocity, and lateral amplitude were also assessed, which showed consistent results (Fig. 2E). These results suggest that selective deletion of *Geminin* from pre-meiotic spermatogonia leads to infertility in mice.

Undifferentiated spermatogonia and spermatocytes are reduced in Geminin-deficient males

To precisely determine which stage of spermatogenesis was affected by *Geminin* knockout, the expression of undifferentiated spermatogonia marker Sall4 [28] and meiotic marker SCP3 [29] was analyzed. Sall4-positive germ cells were detected in the seminiferous tubules of both control and *Geminin*-deficient testes (Fig. 3A), but the number of Sall4-positive germ cells was significantly

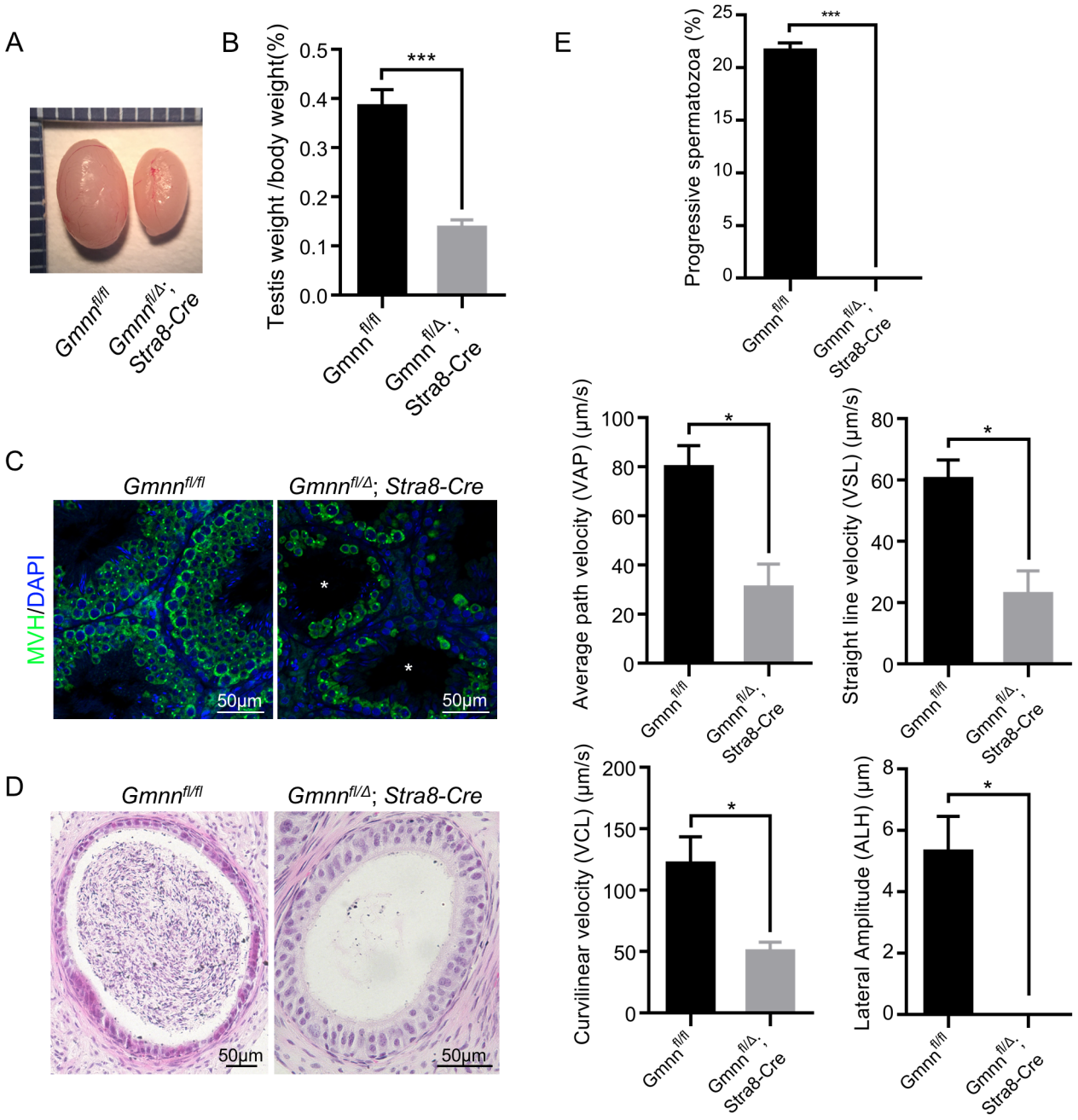

Fig. 2. Deletion of geminin in the testes by *Stra8-Cre* resulted in massive germ cell loss. A, B: The size and the weight of testes from adult *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* mice were significantly smaller than those of control littermates. Testis weight/body weight: *Gmnn*$^{fl/fl}$, 0.39 ± 0.03; *Gmnn*$^{fl/\Delta}$; *Stra8-Cre*, 0.14 ± 0.02, P < 0.001. C: Germ cells were labeled with anti-MVH antibody (green) and DAPI (blue). Markedly fewer MVH-positive germ cells were observed in the seminiferous tubules from *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testis (white asterisks). D: Histological analysis of the caudal epididymides of *Gmnn*$^{fl/fl}$ and *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* mice. E: The percentage of progressive spermatozoa (S.E.M., N = 3, P < 0.001) and sperm motion variables including average path velocity, straight line velocity, curvilinear velocity, and lateral amplitude were assessed (S.E.M., N = 3, P < 0.05). *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* mice showed significantly impaired sperm motility.

reduced in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* tubules compared to in controls (Fig. 3B, P < 0.01). SCP3-positive germ cells were detected in most seminiferous tubules of control testes and were evenly arranged in the seminiferous epithelium layer. In contrast, the number of SCP3-positive germ cells was dramatically reduced in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes based on weaker immunohistochemistry staining (Fig. 3C). Western blot analyses also confirmed that SCP3 was markedly decreased in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes (Fig. 3D). To further

Table 1. Breeding assay of *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* males

Group	Number	Litters/male in 6 months (mean ± S.E.M.)	Pups/litter (mean ± S.E.M.)
Gmnn$^{fl/fl}$	7	9.29 ± 0.63	7.14 ± 0.75
Gmnn$^{fl/\Delta}$; *Stra8-Cre*	6	0	0

explore which stage of germ cells was affected by *Geminin* deletion, PAS and hematoxylin staining were conducted [30]. As shown in Fig. 3E and 3F, the numbers of preleptotene, leptotene, zygotene, pachytene, and diplotene spermatocytes were dramatically reduced. Among these stages, the number of pachytene spermatocytes from *Geminin*-deficient testes was most severely reduced. These findings demonstrate that specific deletion of *Geminin* results reduced the number of both undifferentiated spermatogonia and spermatocytes, and that the pachytene stage was the most severely impaired.

Deletion of Geminin results in impaired proliferation, increased H2AX phosphorylation, and apoptosis

To further examine the functions of *Geminin* in spermatogenesis, immunostaining for the cell proliferation marker proliferating cell nuclear antigen (PCNA) [31] was performed in 8-week-old testis sections. PCNA-positive staining was prominent in control testes, whereas a markedly decreased number of PCNA-positive germ cells was observed in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* mice (Fig. 4A).

It has been reported that *Geminin* deletion induces DNA damage and elevates apoptosis in oocytes and early embryos [14, 16, 32]. Therefore, the expression of phosphorylated H2AX (γH2AX) was examined by immunofluorescence and western blotting in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes. The level of γH2AX was dramatically increased compared to that seen in the control group, showing a stronger fluorescence signal (Fig. 4B). Consistent results were obtained by western blot analysis (Fig. 4C). In addition, numerous TUNEL-positive cells were detected in the seminiferous tubules of *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* mice, but not in control testes (Fig. 4D).

Depletion of Geminin decreases Cdt1 but increases phosphorylation of Chk1 and Chk2 in germ cells

The absence of geminin, an inhibitor of Cdt1, results in accumulation and proteolysis of Cdt1, leading to DNA re-replication and triggering the Chk1/Chk2-dependent checkpoint [32–35]. Western blot analysis of testes showed that Cdt1 was decreased, while the levels of Chk1 and Chk2 phosphorylation were increased (Fig. 5). These results suggest that ablation of *Geminin* from the male germline induces DNA re-replication and activates a cell cycle checkpoint.

Discussion

We found that specific deletion of *Geminin* from pre-meiotic spermatogonia resulted in testicular developmental defects and abnormal spermatogenesis. *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* males showed complete infertility. Fewer Sall4-positive and SCP3-positive germ cells were observed in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes compared to in control males, indicating that both undifferentiated spermatogonia and spermatocytes were affected by ablation of *Geminin*. The decreased number of PCNA-positive germ cells in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes compared to in control males indicates that geminin is required for germline cell proliferation. Changes in the levels of γH2AX, Cdt1 protein, and Chk1/Chk2 phosphorylation suggest that depletion of geminin in germ cells causes DNA damage and damage-induced apoptosis as a result of DNA re-replication, which causes meiotic defects in spermatocytes.

Immunofluorescence results showed that geminin was highly expressed in spermatocytes (Fig. 1B), which is consistent with the results of a previous study [22]. In the study, *Geminin* was selectively eliminated from spermatogonia by using *Vasa-Cre*, which is expressed from E15, resulting in the complete spermatogonial loss of *Gmnn*$^{fl/\Delta}$; *Vasa-Cre* males during the first wave of spermatogenesis by P4. Additionally, geminin is not essential for male germ cell meiosis or spermiogenesis, as revealed by using the *HspA2-Cre*-mediated knockout system. Because expression of *HspA2-Cre* begins in spermatocytes in the leptotene-zygotene stage, in which spermatogonia have already differentiated into primary spermatocytes, the functions of geminin in pre-meiotic DNA replication and subsequent spermatogenesis were unclear. Therefore, in this study, *Gmnn*^{fl} mice were crossed with transgenic mice expressing *Stra8-Cre*, which is detected in early-stage spermatogonia from P3 and becomes stronger at P7 through preleptotene-stage spermatocytes, the developmental stage that occurs around the onset of meiosis [24].

Breeding assays indicated that the lack of geminin caused infertility, but elongating spermatids were observed in seminiferous tubules and epididymides. This may be because of incomplete Cre excision; as reported previously, Cre is only active in a subpopulation of undifferentiated spermatogonia [24]. In addition, spermatogonial stem cells undergo continuous self-renewal and differentiation throughout the male reproductive life [36]. The short expression time of *Stra8* promoter-driven Cre and sustained spermatogenesis led to the production of spermatozoa. Five DNA repair mechanisms [37], such as nucleotide excision repair, have evolved to maintain genomic integrity to compensate for DNA damage in spermatozoa, which may also play a role. As shown in our study, γH2AX accumulation and increased apoptosis were detected. Additionally, notably stronger signals for γH2AX were observed in the elongated spermatids of conditional knockout mice (Fig. 4B, right panel). This suggests that deletion of geminin leads to DNA damage in elongated spermatids and results in motility defects in spermatozoa [38, 39].

Sall4 is a marker for undifferentiated A_s, A_{pr}, and A_{al} spermatogonia, which undergo mitosis [28]. Geminin deletion by the *Stra8-Cre* conditional knockout system begins in Type A spermatogonia [24]. The decreased number of Sall4-positive spermatogonia suggests that geminin is required for the mitotic proliferation of spermatogonia, which we described previously [22]. A reduced number of spermatocytes in all stages was observed in *Geminin*-deficient testes and that of pachytene spermatocytes was decreased most severely among these stages. The mid-pachytene checkpoint occurs at testis epithelial stage IV and pairing problems of aberrant chromosomes leads to apoptosis of these spermatocytes [40, 41]. Pachytene spermatocytes from *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes may therefore undergo apoptosis. A small number of pachytene, diplotene spermatocytes, and round and elongated spermatids were observed in geminin-deficient testes, potentially because of insufficient Cre excision.

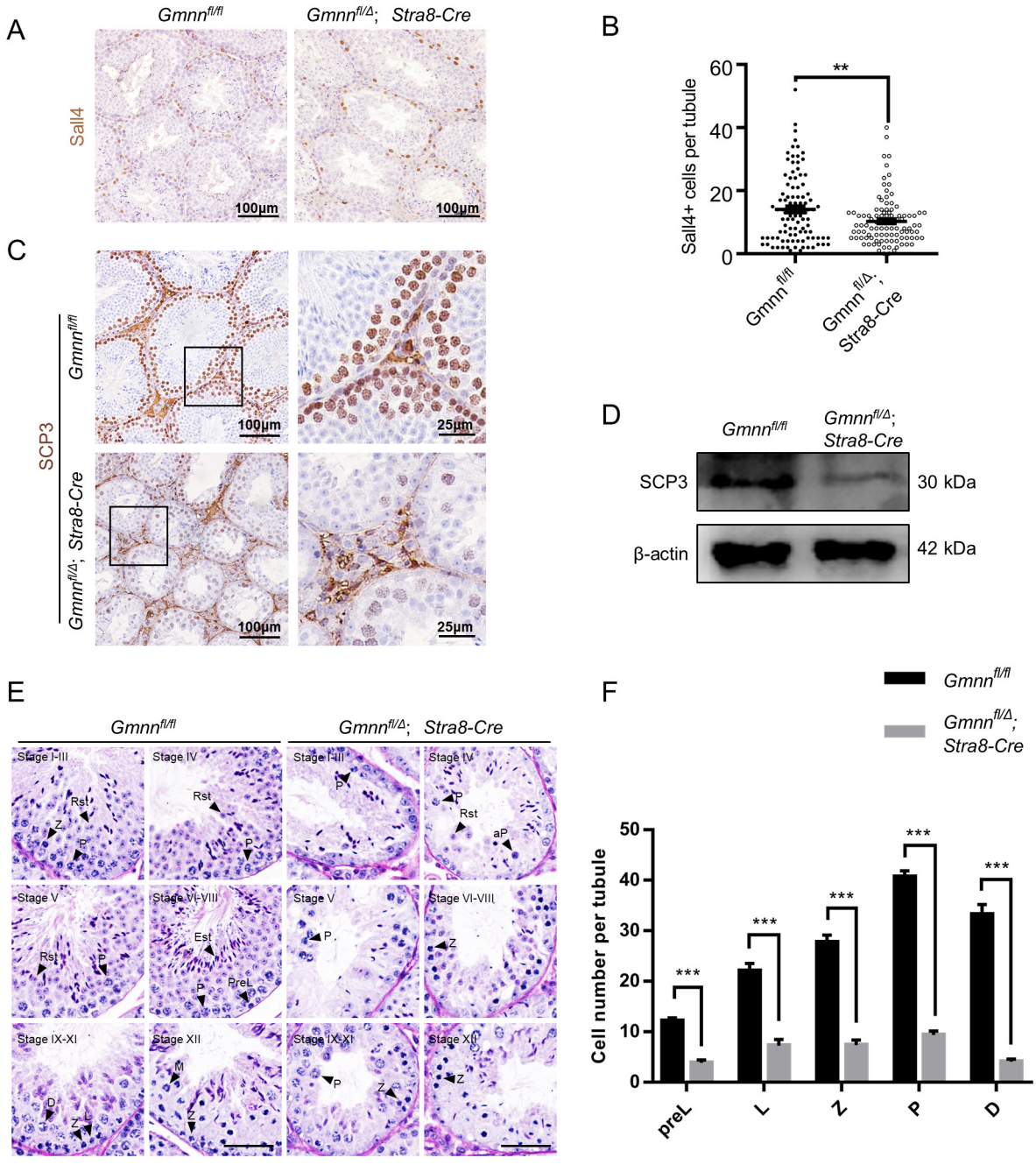

Fig. 3. Undifferentiated spermatogonia and spermatocytes were reduced in *Geminin*-deficient males. A: Sall4 immunohistochemical analysis of germ cells in $Gmnn^{fl/fl}$ and $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice. B: Quantification of Sall4-positive germ cells in the seminiferous tubules of $Gmnn^{fl/fl}$ and $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice. $Gmnn^{fl/fl}$: 14.09 ± 1.09, $Gmnn^{fl/\Delta}$; *Stra8-Cre*: 10.31 ± 0.74, N = 100, P < 0.01. C, D: SCP3 immunohistochemical analysis and western blotting indicated a decrease in SCP3 protein level. E: PAS staining analysis of testis sections in $Gmnn^{fl/fl}$ and $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice. PreL-preleptotene spermatocytes; L-leptotene spermatocytes; Z-zygotene spermatocytes; P-pachytene spermatocytes; D-diplotene spermatocytes; M-meiotic divisions; Rst-round spermatids; Est-elongated spermatids. Scale bar: 50 μm. F: Quantification of preleptotene, leptotene, zygotene, pachytene, diplotene spermatocytes in the seminiferous tubules of in $Gmnn^{fl/fl}$ and $Gmnn^{fl/\Delta}$; *Stra8-Cre* mice.

Previous studies showed that the main role of geminin is to bind to and inhibit Cdt1 protein [6, 33, 42–46], which contributes to genome stability by rigorously controlling genome replication exactly once per cell cycle [47]. Deletion of *Geminin* activates Cdt1 and induces DNA re-replication [48, 49]. The overreplication of DNA then activates the ATM/ATR pathway, which triggers cell cycle checkpoint arrest [48], resulting in increased Chk1/Chk2 phosphorylation, as shown in our study (Fig. 5). The lower Cdt1 level may be a consequence

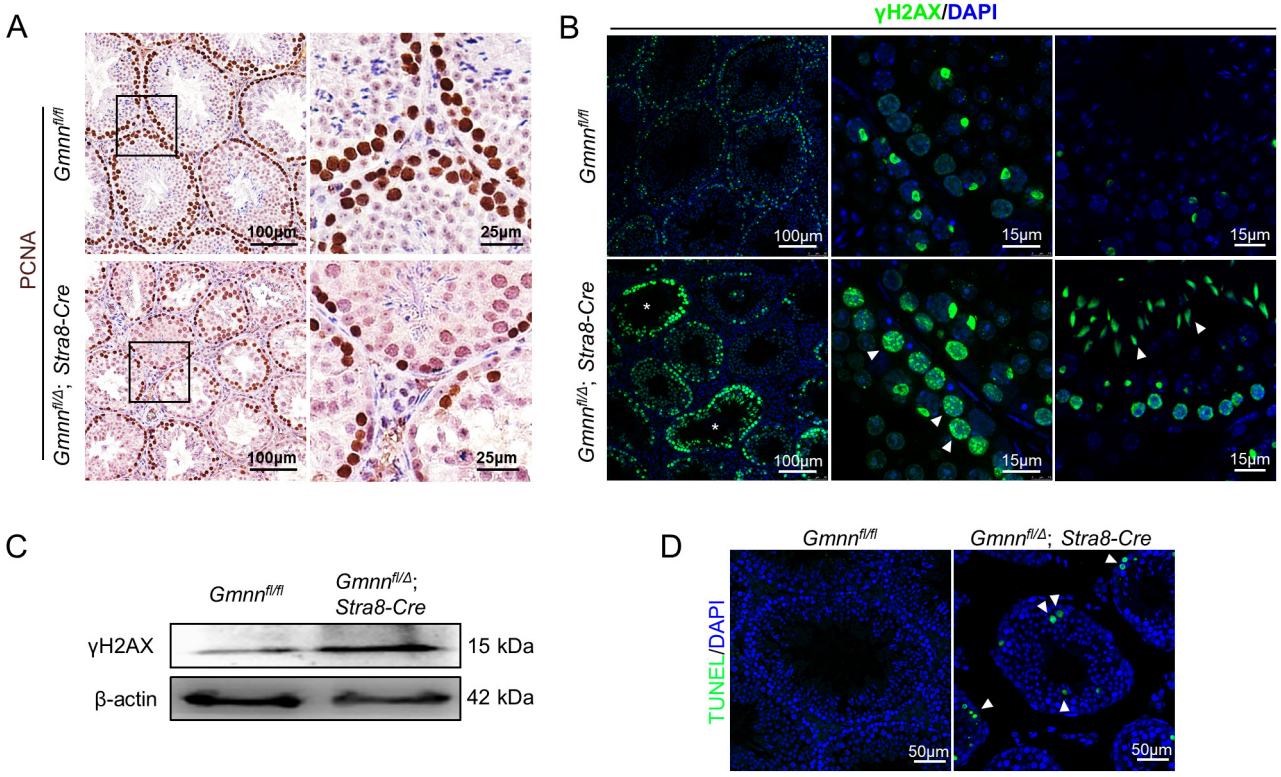

Fig. 4. Deletion of geminin resulted in impaired proliferation, increased H2AX phosphorylation, and elevated apoptosis. A: The number of PCNA-positive germ cells was dramatically reduced. B, C: The expression of γH2AX of control and *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes was examined by immunofluorescence and western blotting. The level of γH2AX (B, green, white arrowheads) was dramatically increased (B, white asterisks). D: The number of apoptotic cells was markedly increased in *Gmnn*$^{fl/\Delta}$; *Stra8-Cre* testes (white arrowheads). Nuclei were stained with DAPI (blue).

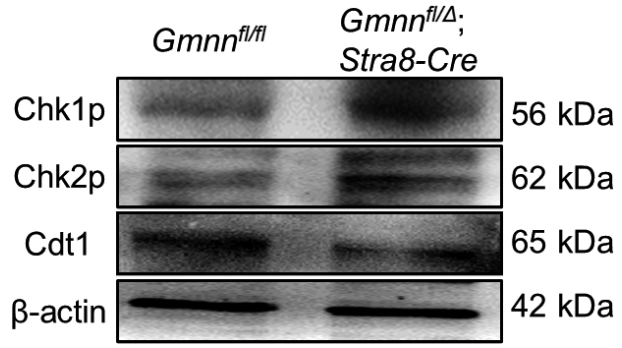

Fig. 5. Deletion of geminin resulted in decreased Cdt1 and increased phosphorylation of Chk1 and Chk2 in germ cells. Western blot analysis of testes showed that Cdt1 protein was decreased, whereas the levels of Chk1 and Chk2 phosphorylation were increased.

of Cdt1 proteolysis [50], possibly because of re-replication-induced DNA damage [51].

In summary, *Stra8-Cre*-driven germline-specific deletion of *Geminin* results in infertility and germ cell loss. Geminin, which is required for accurate DNA replication, is important for both spermatogonial self-renewal and meiosis progression and is essential for DNA damage repair during spermatogenesis.

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References

1. **Kroll KL, Salic AN, Evans LM, Kirschner MW.** Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. *Development* 1998; **125**: 3247–3258. [Medline]
2. **McGarry TJ, Kirschner MW.** Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* 1998; **93**: 1043–1053. [Medline] [CrossRef]
3. **Luo L, Kessel M.** Geminin coordinates cell cycle and developmental control. *Cell Cycle* 2004; **3**: 711–714. [Medline] [CrossRef]
4. **Seo S, Kroll KL.** Geminins double life: chromatin connections that regulate transcription at the transition from proliferation to differentiation. *Cell Cycle* 2006; **5**: 374–379. [Medline] [CrossRef]
5. **Yoshida K.** Geminin organizes the molecular platform to balance cellular proliferation and differentiation. *Front Biosci* 2007; **12**: 2984–2992. [Medline] [CrossRef]
6. **Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A.** Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* 2000; **290**: 2309–2312. [Medline] [CrossRef]

7. **Nishitani H, Taraviras S, Lygerou Z, Nishimoto T.** The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J Biol Chem* 2001; **276**: 44905–44911. [Medline] [CrossRef]
8. **Tada S, Li A, Maiorano D, Méchali M, Blow JJ.** Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat Cell Biol* 2001; **3**: 107–113. [Medline] [CrossRef]
9. **Wong PG, Glozak MA, Cao TV, Vaziri C, Seto E, Alexandrow M.** Chromatin unfolding by Cdt1 regulates MCM loading via opposing functions of HBO1 and HDAC11-geminin. *Cell Cycle* 2010; **9**: 4351–4363. [Medline] [CrossRef]
10. **Seo S, Herr A, Lim JW, Richardson GA, Richardson H, Kroll KL.** Geminin regulates neuronal differentiation by antagonizing Brg1 activity. *Genes Dev* 2005; **19**: 1723–1734. [Medline] [CrossRef]
11. **Yellajoshyula D, Patterson ES, Elitt MS, Kroll KL.** Geminin promotes neural fate acquisition of embryonic stem cells by maintaining chromatin in an accessible and hyperacetylated state. *Proc Natl Acad Sci USA* 2011; **108**: 3294–3299. [Medline] [CrossRef]
12. **Roukos V, Iliou MS, Nishitani H, Gentzel M, Wilm M, Taraviras S, Lygerou Z.** Geminin cleavage during apoptosis by caspase-3 alters its binding ability to the SWI/SNF subunit Brahma. *J Biol Chem* 2007; **282**: 9346–9357. [Medline] [CrossRef]
13. **Yang VS, Carter SA, Hyland SJ, Tachibana-Konwalski K, Laskey RA, Gonzalez MA.** Geminin escapes degradation in G1 of mouse pluripotent cells and mediates the expression of Oct4, Sox2, and Nanog. *Curr Biol* 2011; **21**: 692–699. [Medline] [CrossRef]
14. **Hara K, Nakayama KI, Nakayama K.** Geminin is essential for the development of preimplantation mouse embryos. *Genes Cells* 2006; **11**: 1281–1293. [Medline] [CrossRef]
15. **Patterson ES, Waller LE, Kroll KL.** Geminin loss causes neural tube defects through disrupted progenitor specification and neuronal differentiation. *Dev Biol* 2014; **393**: 44–56. [Medline] [CrossRef]
16. **Ma XS, Lin F, Wang ZW, Hu MW, Huang L, Meng TG, Jiang ZZ, Schatten H, Wang ZB, Sun QY.** Geminin deletion in mouse oocytes results in impaired embryo development and reduced fertility. *Mol Biol Cell* 2016; **27**: 768–775. [Medline] [CrossRef]
17. **Sharpe RS**. Regulation of spermatogenesis. *In:* E Knobil JN (ed.), The Physiology of Reproduction. New York: Raven; 1994.
18. **Oatley JM, Brinster RL.** Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol* 2008; **24**: 263–286. [Medline] [CrossRef]
19. **de Rooij DG, Russell LD.** All you wanted to know about spermatogonia but were afraid to ask. *J Androl* 2000; **21**: 776–798. [Medline]
20. **Russell L, Ettin R, Sinhahikim A, Clegg E.** Histological and Histopathological Evaluation of the Testis. St. Louis, MO: Cache River Press; 1990.
21. **Nussbaum R, McInnes RR, Willard HF, Hamosh A.** Genetics in Medicine. Thompson & Thompson; 2007.
22. **Barry KA, Schultz KM, Payne CJ, McGarry TJ.** Geminin is required for mitotic proliferation of spermatogonia. *Dev Biol* 2012; **371**: 35–46. [Medline] [CrossRef]
23. **Inselman AL, Nakamura N, Brown PR, Willis WD, Goulding EH, Eddy EM.** Heat shock protein 2 promoter drives Cre expression in spermatocytes of transgenic mice. *Genesis* 2010; **48**: 114–120. [Medline]
24. **Sadate-Ngatchou PI, Payne CJ, Dearth AT, Braun RE.** Cre recombinase activity specific to postnatal, premeiotic male germ cells in transgenic mice. *Genesis* 2008; **46**: 738–742. [Medline] [CrossRef]
25. **Shinnick KM, Eklund EA, McGarry TJ.** Geminin deletion from hematopoietic cells causes anemia and thrombocytosis in mice. *J Clin Invest* 2010; **120**: 4303–4315. [Medline] [CrossRef]
26. **Zhang D, Ma W, Li YH, Hou Y, Li SW, Meng XQ, Sun XF, Sun QY, Wang WH.** Intraoocyte localization of MAD2 and its relationship with kinetochores, microtubules, and chromosomes in rat oocytes during meiosis. *Biol Reprod* 2004; **71**: 740–748. [Medline] [CrossRef]
27. **Gonzalez MA, Tachibana KE, Adams DJ, van der Weyden L, Hemberger M, Coleman N, Bradley A, Laskey RA.** Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes Dev* 2006; **20**: 1880–1884. [Medline] [CrossRef]
28. **Gassei K, Orwig KE.** SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. *PLoS ONE* 2013; **8**: e53976. [Medline] [CrossRef]
29. **Ball RL, Fujiwara Y, Sun F, Hu J, Hibbs MA, Handel MA, Carter GW.** Regulatory complexity revealed by integrated cytological and RNA-seq analyses of meiotic substages in mouse spermatocytes. *BMC Genomics* 2016; **17**: 628. [Medline] [CrossRef]
30. **Ahmed EA, de Rooij DG.** Staging of mouse seminiferous tubule cross-sections. *Meiosis: Volume 2, Cytological Methods* 2009: 263−277.
31. **Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, *et al*** Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol* 1990; **162**: 285–294. [Medline] [CrossRef]
32. **Kerns SL, Schultz KM, Barry KA, Thorne TM, McGarry TJ.** Geminin is required for zygotic gene expression at the Xenopus mid-blastula transition. *PLoS ONE* 2012; **7**: e38009. [Medline] [CrossRef]
33. **Narasimhachar Y, Coué M.** Geminin stabilizes Cdt1 during meiosis in Xenopus oocytes. *J Biol Chem* 2009; **284**: 27235–27242. [Medline] [CrossRef]
34. **Kerns SL, Torke SJ, Benjamin JM, McGarry TJ.** Geminin prevents rereplication during xenopus development. *J Biol Chem* 2007; **282**: 5514–5521. [Medline] [CrossRef]
35. **McGarry TJ.** Geminin deficiency causes a Chk1-dependent G2 arrest in Xenopus. *Mol Biol Cell* 2002; **13**: 3662–3671. [Medline] [CrossRef]
36. **Yamada M, De Chiara L, Seandel M.** Spermatogonial stem cells: implications for genetic disorders and prevention. *Stem Cells Dev* 2016; **25**: 1483–1494. [Medline] [CrossRef]
37. **Gunes S, Al-Sadaan M, Agarwal A.** Spermatogenesis, DNA damage and DNA repair mechanisms in male infertility. *Reprod Biomed Online* 2015; **31**: 309–319. [Medline] [CrossRef]
38. **Evenson DP, Larson KL, Jost LK.** Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 2002; **23**: 25–43. [Medline] [CrossRef]
39. **Giwercman A, Richthoff J, Hjøllund H, Bonde JP, Jepson K, Frohm B, Spano M.** Correlation between sperm motility and sperm chromatin structure assay parameters. *Fertil Steril* 2003; **80**: 1404–1412. [Medline] [CrossRef]
40. **Ashley T, Westphal C, Plug-de Maggio A, de Rooij DG.** The mammalian midpachytene checkpoint: meiotic arrest in spermatocytes with a mutation in Atm alone or in combination with a Trp53 (p53) or Cdkn1a (p21/cip1) mutation. *Cytogenet Genome Res* 2004; **107**: 256–262. [Medline] [CrossRef]
41. **de Rooij DG, de Boer P.** Specific arrests of spermatogenesis in genetically modified and mutant mice. *Cytogenet Genome Res* 2003; **103**: 267–276. [Medline]
42. **Benjamin JM, Torke SJ, Demeler B, McGarry TJ.** Geminin has dimerization, Cdt1-binding, and destruction domains that are required for biological activity. *J Biol Chem* 2004; **279**: 45957–45968. [Medline] [CrossRef]
43. **Lee C, Hong B, Choi JM, Kim Y, Watanabe S, Ishimi Y, Enomoto T, Tada S, Kim Y, Cho Y.** Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. *Nature* 2004; **430**: 913–917. [Medline] [CrossRef]
44. **Saxena S, Yuan P, Dhar SK, Senga T, Takeda D, Robinson H, Kornbluth S, Swaminathan K, Dutta A.** A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. *Mol Cell* 2004; **15**: 245–258. [Medline] [CrossRef]
45. **Li A, Blow JJ.** Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus. *EMBO J* 2005; **24**: 395–404. [Medline] [CrossRef]
46. **Lutzmann M, Maiorano D, Méchali M.** A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus. *EMBO J* 2006; **25**: 5764–5774. [Medline] [CrossRef]
47. **Arias EE, Walter JC.** Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes Dev* 2007; **21**: 497–518. [Medline] [CrossRef]
48. **Lin JJ, Dutta A.** ATR pathway is the primary pathway for activating G2/M checkpoint induction after re-replication. *J Biol Chem* 2007; **282**: 30357–30362. [Medline] [CrossRef]
49. **Rakotomalala L, Studach L, Wang WH, Gregori G, Hullinger RL, Andrisani O.** Hepatitis B virus X protein increases the Cdt1-to-geminin ratio inducing DNA re-replication and polyploidy. *J Biol Chem* 2008; **283**: 28729–28740. [Medline] [CrossRef]
50. **Ballabeni A, Melixetian M, Zamponi R, Masiero L, Marinoni F, Helin K.** Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. *EMBO J* 2004; **23**: 3122–3132. [Medline] [CrossRef]
51. **Hall JR, Lee HO, Bunker BD, Dorn ES, Rogers GC, Duronio RJ, Cook JG.** Cdt1 and Cdc6 are destabilized by rereplication-induced DNA damage. *J Biol Chem* 2008; **283**: 25356–25363. [Medline] [CrossRef]